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AUTHOR(S):

Kuwabara, Takashige; Mori, Kiyoshi; Mukoyama, Masashi; Kasahara, Masato; Yokoi, Hideki; Saito, Yoko; Yoshioka, Tetsuro; ... Sugawara, Akira; Barasch, Jonathan; Nakao, Kazuwa

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Source and changes of urinary Ngal in renal injury

[Original Article]

[Title]

Urinary neutrophil gelatinase-associated lipocalin levels reflect damage in glomeruli, proximal and distal nephrons**T Kuwabara¹, K Mori¹, M Mukoyama¹, M Kasahara^{1,2}, H Yokoi¹, Y Saito¹, T Yoshioka¹, Y Ogawa¹, H Imamaki¹, T Kusakabe¹, K Ebihara¹, M Omata³, N Satoh⁴, A Sugawara⁵, J Barasch⁶ and K Nakao¹.****¹Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, Japan;****²Division of Nephrology, Kobe City General Hospital, Kobe, Japan;****³Biomedical Research Laboratories, Asubio Pharma Co. Ltd., Osaka, Japan;****⁴Clinical Research Institute for Endocrine Metabolic Diseases, National Hospital Organization, Kyoto Medical Center, Kyoto, Japan;****⁵Department of Nephrology, Kyoto Medical Center, Kyoto, Japan;****⁶Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, NY, USA.****Correspondence:** *K Mori, Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine; 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.**E-mail: keyem@kuhp.kyoto-u.ac.jp***KEYWORDS:** diabetic nephropathy, nephrotic syndrome, obstructive nephropathy; acute renal failure; albuminuria

[Running Headline]

Source and changes of urinary Ngal in renal injury

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ABSTRACT

Urinary (u) neutrophil gelatinase-associated lipocalin (Ngal or lipocalin 2) is a very early and sensitive biomarker for kidney injury but its source and time course during treatment remain elusive. In the present study we show that uNgal concentrations were markedly elevated in A-ZIP/F-1 lipoatrophic diabetes and streptozotocin-induced diabetic (STZ) mice. In STZ mice, reabsorption of Ngal in the proximal tubule was severely reduced but upregulation of Ngal mRNA and protein expression in the kidney was little compared to non-diabetic control mice, indicating that increased uNgal excretion derived mainly from impaired renal reabsorption. In mouse kidneys treated with unilateral ureteral obstruction, Ngal protein was highly accumulated in thick ascending limbs of Henle and in the urine pooled in pelvis. In 5 patients with nephrotic syndrome or interstitial nephritis, uNgal levels were elevated markedly, and were decreased in response to treatment including steroids. Treatment of STZ mice with angiotensin receptor blocker candesartan dramatically reduced uNgal excretion. These findings indicate that uNgal level is a unique biomarker of renal injury incorporating information of glomerular filtration defect, tubular reabsorption dysfunction and distal nephron damage, and may be useful in monitoring disease activity and treatment efficacy in various forms of renal injury.

INTRODUCTION

Neutrophil gelatinase-associated lipocalin (Ngal) is a differentiation inducer for epithelia in embryonic kidney, whose expression is dramatically increased in acute kidney injury (AKI).¹⁻⁵ Ngal exerts a spectrum of iron-dependent biological activities,^{1-4,6} and administration of Ngal protein mitigates renal injury in mice, suggesting that functional consequence of Ngal upregulation is renoprotection.² Ngal mRNA levels in the kidney are increased as much as by 1000-fold during renal ischemia-reperfusion injury in mice.^{2,7} Ngal protein starts to accumulate within a few hours in the blood and urine during AKI.⁸⁻¹¹ These characteristics of Ngal have made it a promising biomarker of AKI that is found in the blood and urine.^{1,7,12-15} Furthermore, several studies reported that serum and urinary Ngal levels are elevated proportionally to the extent of renal damage in chronic kidney disease (CKD),^{16,17} but the source and the time course of urinary Ngal concentrations are largely unknown. In this study, we investigated urinary Ngal levels in four types of renal damage caused by distinct mechanism: nephrotic syndrome caused by glomerular disorders, diabetic nephropathy, obstructive nephropathy and interstitial nephritis. We also examined whether measurement of urinary Ngal is useful for the monitoring of renal damage in mice or humans during the treatment course.

RESULTS

Urinary Ngal excretion is proportional to albumin excretion in mouse models of diabetic nephropathy

As a model of diabetic nephropathy, we first examined urinary Ngal concentrations in A-ZIP/F-1 transgenic mice, which are characterized with lipotrophic diabetes, fatty liver, hyperlipidemia, severe insulin resistance and massive proteinuria.¹⁸⁻²⁰ Urinary Ngal excretion in A-ZIP/F-1 mice at 10 months of age was much larger than that in control FVB/N mice (Figure 1). By Western blot, we observed 30 and 25 kDa bands with Ngal immunoreactivity, and the larger band was found only in the urine from A-ZIP/F-1 mice and not in the urine or tissues from normal, diabetic or obstructed kidneys of mice with C57BL/6 background (see below). The larger protein may have heavier glycosylation than the smaller protein.²¹ When the amounts of two proteins were added, mice with larger urinary Ngal levels tended to have larger urinary albumin levels.

Next, we studied streptozotocin (STZ)-induced diabetes, which manifests with insulin deficiency and microalbuminuria. In STZ mice, urinary albumin excretion increased gradually and, after 8 weeks, reached 7.8-fold of the level before STZ injection (Figure 2). On the other hand, urinary Ngal levels were elevated by 77-fold at 8 weeks. The extent of Ngal and albumin excretion was highly variable among different mice, but urinary albumin levels and log transformation of Ngal levels showed a close linear correlation throughout the course of 12 week observation period.

Elevation of urinary Ngal excretion in STZ mice is not caused by renal synthesis but by reabsorption defect, and treatment with angiotensin receptor blocker reduces urinary Ngal levels

To examine whether local expression of Ngal is increased in STZ mouse kidneys, we studied expression levels of Ngal protein in the whole kidney preparation of STZ and non-STZ control mice and found no significant difference at 8 weeks after STZ treatment (Figure 3). We did not find significant alteration of Ngal protein expression in the livers, either. Of note, serum Ngal levels in STZ mice were significantly lower than those in non-STZ mice (23 ± 5 versus 111 ± 22 ng ml⁻¹, $n=3-4$, $P<0.01$). We measured Ngal mRNA expression levels in the kidneys and livers of STZ mice, but they were increased only marginally compared to control mice. As a positive control, Ngal mRNA expression was increased by 100 fold in obstructed kidneys after 1 day of ureter ligation ($P<0.001$). These findings suggested that dramatic (nearly 80 fold) increase of urinary Ngal excretion in STZ mice cannot be explained by augmentation of Ngal protein synthesis in the kidney, and led us to investigate tubular reabsorption of Ngal. Injection of histidine-tagged or Alexa Fluor 546-labeled Ngal (21 kDa in size) in the peritoneum of non-STZ mice resulted in glomerular filtration and efficient reabsorption of Ngal at the proximal tubules from the apical side, thus no exogenous Ngal was detected in the urine (Figure 4). In STZ mice, on the other hand, substantial amount of exogenous Ngal was

excreted in the urine and reabsorption of labeled Ngal was reduced by 47 % ($P < 0.01$).

Since treatment of diabetic nephropathy with angiotensin receptor blocker (ARB) reduces proteinuria and ameliorates renal injury,²² we gave the ARB candesartan to STZ mice through drinking water at 10 mg per kg per day (Figure 5). After one week, urinary albumin levels were decreased by 14 % ($P < 0.05$) and urinary Ngal levels were decreased by 77 % ($P < 0.05$). Serum Ngal levels were not altered by candesartan (20 ± 6 ng ml⁻¹). The dose of candesartan used was a subdepressor dose, and did not significantly affect body weights, blood glucose, urea nitrogen and creatinine levels (Table 1). Through these findings, we conclude that increased urinary Ngal excretion in STZ mice was caused mainly by reabsorption defect and treatment with candesartan partially normalized urinary Ngal levels.

Urinary Ngal levels are highly elevated in human cases of nephrotic syndrome and are decreased in response to treatment

As cases of glomerular disorders with nephrotic syndrome, we investigated the clinical courses and changes in serum and urinary Ngal levels in human subjects. Case 1 was a 68-year-old female with biopsy-proven minimal change disease. She had nephrotic range proteinuria (14 g day⁻¹) and gained body weight by 15 kg (from 62 to 77 kg) within 3 weeks. She was treated with iv steroid pulse (methyl prednisolone 1 g \times 3 days), followed with oral prednisolone (beginning with 35 mg per day), and with three courses of hemodialysis on days 8, 9 and 12 after admission (Figure 6). Her proteinuria, edema and azotemia resolved gradually. Concomitantly, urinary and serum Ngal levels decreased during the treatment, but reduction was much faster for urinary Ngal levels. There was a temporal elevation of urinary Ngal levels on day 21, which might reflect the reappearance of oligouria or proteinuria.

Case 2 was a 26-year-old female, who was diagnosed to have membranous-type lupus nephritis (ISN/RPS class V). She was treated with two courses of iv steroid pulse, followed with oral prednisolone (Figure 6). Her urinary levels of protein and Ngal were decreased sharply within 17 days.

Case 3 was a 55-year-old female with clinically-diagnosed lupus nephritis (no biopsy). She had been treated with two courses of iv steroid pulse (1 g \times 3 days) and oral prednisolone had been tapered from 50 to 35 mg per day, before changing the hospitals to ours. She was given iv cyclophosphamide pulse (0.4 g, once, 6 days after admission) and the immunosuppressant mizoribine (Asahi Kasei Pharma, Osaka, Japan; 100 mg per day). Urinary excretion of protein and Ngal decreased slowly (Supplementary Figure S1). Serum creatinine levels were constant throughout the course (0.4-0.5 mg per 100 ml).

Case 4 was a 69-year-old male, who suffered from rapidly-progressing, crescentic glomerulonephritis accompanied with moderate tubulointerstitial damage. His serum contained myeloperoxidase-type anti-neutrophil cytoplasmic antibody

(MPO-ANCA, 138 EU). The maximum serum creatinine level was 5.6 mg per 100 ml, and the proteinuria and azotemia responded slowly to treatment containing oral and iv steroid and iv cyclophosphamide (Supplementary Figure S1). Macrohematuria was observed, peaking at 5 days after cyclophosphamide administration. After 10 months, he showed signs of recurrence which were worsening in proteinuria, hematuria, azotemia and MPO-ANCA titer (from <10 to 37 EU). We observed re-elevation of urinary Ngal levels during acute worsening of nephritis. Cyclosporine A (75 mg day⁻¹ before breakfast) was added at 13 months, which appeared to support reduction of above mentioned signs.

To understand renal localization of Ngal protein in nephrotic patients, we carried out immunofluorescence study of renal biopsy samples and found close co-localization of signals of Ngal and albumin at the apical side of tubules. The results for Case 1 are shown in Figure 7. These findings are consistent with those in animal experiments shown above (Figure 4), indicating highly active reabsorption of Ngal from glomerular filtrate.

In mice with obstructive nephropathy, Ngal protein is specifically located in the distal nephrons in the obstructed side by local synthesis, whereas it is confined to the proximal tubules in the contra-lateral side of the kidneys by reabsorption

As a model of post-renal kidney injury, we investigated mice with unilateral ureteral obstruction (UUO), in which distal nephrons are primarily affected, and studied the changes of Ngal protein levels in the obstructed and non-obstructed sides of the kidneys, serum, and urine (Figures 7, 8). We also determined the renal distribution of Ngal by immunohistochemistry along with nephron segment markers: aquaporin (AQP) 1 for proximal tubules, Tamm-Horsfall protein (THP) for thick ascending limbs of Henle, and AQP 2 for collecting ducts (Figure 7, Supplementary Figures S2-S4). In the UUO kidneys, after 1 day of ureter ligation, Ngal protein was expressed exclusively in thick ascending limbs of Henle, which was also expressing THP and was prominently dilated, suggesting that Ngal was synthesized in damaged epithelia. By striking contrast, in the contra-lateral kidneys, Ngal protein was confined to the apical side of aquaporin 1⁺ proximal tubules. Ngal protein levels in the obstructed kidneys and in the urine from the dilated pelvis were continuously elevated for 2 weeks, whereas those in the non-obstructed kidneys and in the serum peaked at day 1 and decreased gradually. A smaller (17 kDa) fragment detected in the kidneys (but not in the urine and serum) using polyclonal anti-Ngal antibody may have been generated by lysosomal proteolysis of Ngal in the tubules.² Ngal mRNA expression was elevated by 100-fold in the obstructed kidneys (Figure 3), but it was only elevated by 3-fold in the contra-lateral kidneys at day 1 (data not shown). These findings indicate that Ngal was synthesized *de novo* in distal nephrons of obstructed kidneys, while it was highly but transiently

accumulated in the serum, filtrated and reabsorbed in the contra-lateral kidneys.

In a case with interstitial nephritis, urinary Ngal levels decreased more rapidly than classic markers of tubular injury.

Next, we investigated whether urinary Ngal is useful for evaluation of renal disorder with low level proteinuria. Case 5 was a 25-year-old male, who was admitted to our hospital, presenting with general malaise, proteinuria (0.4 g day^{-1}), renal glucosuria and mild azotemia. He had taken an over-the-counter cold medicine 6 weeks earlier, and was positive in lymphocyte stimulation test for the drug. Renal biopsy revealed subacute interstitial nephritis with minor glomerular lesions. His signs and symptoms resolved by oral and iv prednisolone treatment. Table 2 and Supplementary Figure S5 summarize the clinical course and changes in urinary biomarkers. The time to 50% reduction of urinary markers was in the order of $\text{Ngal} \leq (\text{total}) \text{ protein} < \alpha 1\text{-}$ and $\beta 2\text{-microglobulins}$ (classic markers of tubular proteinuria) $< \text{N-acetyl-beta-D-glucosaminidase (NAG)}$. The fold-change during treatment was also largest in urinary Ngal, suggesting that urinary Ngal may be useful in monitoring the activity of non-glomerular renal disorders.

DISCUSSION

In the present study, we investigated urinary Ngal concentrations in patients with nephrotic syndrome (caused by acute and severe glomerular disorders) and interstitial nephritis and in mouse models of diabetic or obstructive nephropathy and found that the levels were unequivocally elevated (over 10 fold of control). In diabetic mice induced by STZ (as a model of slowly-progressive CKD), urinary Ngal appeared to derive mostly from impaired reabsorption in proximal tubules. In obstructed kidneys (as a model of post-renal AKI), Ngal was highly expressed in distal nephrons and accumulated in the urine collected from the pelvis. Therefore, STZ-diabetic and obstructed kidneys are the two extreme examples in which the primary source of urinary Ngal is glomerular filtrate and renal synthesis, respectively. In human renal disorders, urinary Ngal should be a mixture of these two major components. These findings indicate that in a variety of kidney diseases, urinary Ngal is a biomarker that can reflect damage in glomeruli, proximal tubules and distal nephrons.

Cross-sectional studies published so far have elucidated that urinary Ngal levels show certain correlation with urinary protein levels.^{16,23} To our knowledge, this is the first human report to show very rapid and simultaneous reduction of urinary Ngal and protein concentrations by medical intervention. Surprisingly, the time course of urinary Ngal levels was associated to that of urinary protein levels not only in diabetic nephropathy and minimal change disease but also in crescentic glomerulonephritis and interstitial nephritis. In the latter disorders, treatment with steroid and immunosuppressant may have ameliorated Ngal reabsorption impairment and epithelial Ngal synthesis at the same time. The present findings suggest that urinary Ngal may be useful in the monitoring of disease activity and treatment efficacy. Of note, we cannot over-generalize the findings in this study to all renal disorders, especially because we did not examine patients with severe, acute tubular necrosis, for instance, caused by renal ischemia or nephrotoxins (in which Ngal is abundantly synthesized by renal epithelia).²

In diabetic nephropathy, albumin excretion is increased by leakage from glomerular filtration barrier.²⁴ On the other hand, a number of reports elucidated the involvement of tubular dysfunction as a cause of albuminuria.²⁴⁻²⁷ The size of Ngal protein (25 kDa) is smaller than albumin and, in normal conditions, Ngal is rapidly filtered by glomeruli and reabsorbed very efficiently by proximal tubules, leaving only 0.1-0.2% in the urine.² By analyzing renal handling of exogenously injected Ngal, the present study emphasized the existence of tubular reabsorption impairment in diabetic nephropathy.²⁸ In reverse, cellular stress in the proximal tubules may cause deterioration in diabetic nephropathy, since it was reported that transgenic overexpression of catalase in the proximal tubules of mice attenuated development of hypertension and albuminuria associating diabetic nephropathy.²⁹

Treatment of STZ-diabetic mice with ARB significantly reduced urinary

excretion of both Ngal and albumin. Two major scenarios can be proposed. First, ARB directly improved reabsorption efficiency in the proximal tubules, which may be mediated by increased peritubular capillary blood flow³⁰ and by amelioration of oxidative stress.³¹ Second, ARB reduced intraglomerular hypertension and hyperfiltration,³² reduced albumin leakage from glomeruli, and the total amount of the ligands for the common scavenger receptor megalin was decreased in the tubular lumen. This may increase the ratio of Ngal and albumin endocytosed at proximal tubules.

Here we used high-dose STZ to induce a model of diabetic nephropathy. Direct toxicity of STZ to proximal tubules may have exaggerated the elevation of urinary Ngal levels in these mice (which is 77-fold of control mice).³³ Hyperfiltration is reported in rodents given STZ.³² In a previous report, 3-fold elevation in creatinine clearance was observed in C57BL/6 mice after treatment with STZ, but serum creatinine levels were not significantly decreased, likely due to concomitant osmotic diuresis and dehydration.³⁴ In the present study, hyperfiltration may have similarly occurred in STZ-diabetic mice. Growth arrest and leanness of STZ-diabetic mice may be partly related to selective reduction of serum Ngal levels (but not serum creatinine and BUN levels) in the present study, since obesity has been shown to be associated with elevated circulating Ngal levels.³⁵

We also observed abundant urinary Ngal excretion in 4 patients with nephrotic syndrome. The mechanism may involve direct competition between Ngal and albumin at the surface of megalin molecule for receptor-mediated endocytosis^{26,36} and also general malfunctioning of proximal tubules because of protein overload. Furthermore, a fraction of urinary Ngal may originate from renal synthesis in addition to reabsorption defect, but Ngal gene expression in human samples was not investigated in this study. The time required for urinary Ngal reduction was variable among cases: ranging from 2 weeks (cases 1 and 2) to more than a month (cases 3 and 4).

In a case with interstitial nephritis, urinary Ngal showed the largest fold increase and the quickest response to steroid treatment in comparison to other urinary biomarkers: total protein, α 1- and β 2-microglobulins and NAG. These findings suggest that urinary Ngal might be particularly useful in the evaluation of kidney recovery in patients with low grade proteinuria.

To summarize, urinary Ngal is a rapid biomarker of kidney injury and recovery showing a large fold-increase or decrease during clinical course of various renal disorders. Proteinuria seems to be one of important factors affecting Ngal'uria.

MATERIALS AND METHODS

Animal experiments

All animal experiments were conducted in accordance with our institutional guidelines for animal research. Male A-ZIP/F-1 heterozygous transgenic mice and control FVB/N littermates were used at 10 month of age, when A-ZIP/F-1 mice exhibit diabetic nephropathy with massive proteinuria.¹⁸⁻²⁰ Other animal experiments were carried out with male C57BL/6J mice (Japan Clea, Tokyo, Japan) starting at 8 week of age, when they weighted 21-23 g. Diabetes was induced by intraperitoneal injection of STZ (180 mg kg⁻¹ of body weight; Sigma, St. Louis, MO, USA) in citrate buffer (pH 4.6) and control mice received only citrate buffer. Blood pressure was measured by the indirect tail-cuff method with MK-2000ST (Muromachi Kikai, Tokyo, Japan). Urine samples were collected with metabolic cages. Urinary albumin was measured with murine albumin ELISA (Exocell; Philadelphia, PA, USA). Serum and urinary creatinine levels were assayed by the enzymatic method (SRL, Tokyo, Japan). This method gives reliable measurement when compared to high-performance liquid chromatography method, even in low concentration materials, and performs much better than Jaffe's colorimetric method.³⁷ Blood glucose and HbA1c levels were determined in tail vein blood at ad libitum-fed conditions using Glutest Ace (Sanwa Kagaku, Nagoya, Japan) and DCA2000+ Analyzer (Bayer Medical, Tokyo, Japan), respectively. Mice were killed under pentobarbital anesthesia before organ collection. Prodrug of candesartan, candesartan cilexetil (TCV-116; Takeda Chemical Industries, Osaka, Japan), was initially dissolved at 10 mg ml⁻¹ in a solution containing 16% polyethylene glycol #300 (vol/vol; Nacalai, Kyoto, Japan), 16% ethanol (Nacalai) and 0.7 M Na₂CO₃, and further diluted in drinking water to be given at a final dose of 10 mg per kg per day. This treatment method³⁸ gave less blood pressure lowering effects compared to gavage administration as previously described.³⁹ For UUO, mice were anesthetized with pentobarbital, the left kidney was exposed by midline incision, and the left ureter was ligated with 4-0 silk at two points.⁴⁰ Mice were sacrificed 1-14 days after the operation.

Recombinant Ngal injection and detection

To investigate renal reabsorption of Ngal, 200 µg of 6xhistidine-tagged (at the carboxyl terminus) or 60 µg of Alexa Fluor 546 (Molecular Probes, Eugene, OR, USA)-labeled recombinant mouse Ngal (expressed in BL21 strain of *E. coli*)² was injected into the peritoneum of mice. Urine samples were collected for 12 hours after His-tagged Ngal injection. Urinary excretion of administered His-tagged Ngal was evaluated by Western blot analysis described below with anti-His antibody (MBL, Nagoya, Japan) or with goat polyclonal anti-mouse Ngal antibody (R&D Systems, Minneapolis, MN, USA). Kidneys were harvested 30 minutes after Alexa Fluor 546-labeled Ngal injection, snap frozen, sliced at 10 µm thickness and examined by a fluorescence microscope (IX81-PAFM; Olympus, Tokyo, Japan). The signal positive areas were measured using MetaMorph 7.5 software (Molecular Devices, Downingtown, PA, USA)

Patients and measurement of human Ngal

Patients who admitted to Kyoto University Hospital for the diagnosis and treatment of renal disorders were enrolled under informed consent. This study was approved by the ethical committee on human research of Kyoto University Graduate School of Medicine. Ngal concentrations in the human serum and urine were determined by sandwich ELISA (AntibodyShop, Gentofte, Denmark) usually after 1000 and 250-fold dilution, respectively.

Western blot analysis

Urine, serum and proteins extracted from organs were separated by SDS-PAGE, transferred onto PVDF membranes, incubated with primary antibody and detected with peroxidase-conjugated secondary antibody and chemiluminescence. Serum was passed through 100-kDa cut-off membrane (Microcon YM-100, Millipore, Bedford, MA, USA) to remove immunoglobulins before analysis.² The amount of Ngal protein was measured by densitometry. Known amounts of recombinant mouse Ngal protein were used as standards.²

Real-time reverse transcription PCR

Total RNA was extracted from mouse kidneys and livers with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA in each sample was synthesized by High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The mRNA levels of Ngal were determined using Premix Ex Taq (Takara Bio, Otsu, Japan) and ABI Prism 7300 Sequence Detector with the following primers and probe: Ngal forward, 5'-ggcagctttacgatgtacagca-3'; Ngal reverse, 5'-tctgatccagtagcgacagcc-3'; Ngal probe, 5'-FAM-catcctggctcagggaccaggaccag-TAMRA-3'. Expression levels of Ngal were normalized by GAPDH (internal control) levels, whose primer-probe set was purchased from Applied Biosystems.⁴¹ Standard curve was made by serial dilution of cDNA from UUO kidneys.

Immunofluorescence of Ngal and albumin

Snap frozen human biopsy samples were sliced at 3 μ m thickness, fixed with acetone, and incubated with a solution containing both FITC-labeled rabbit anti-human albumin antibody (Dako, Glostrup, Denmark) and goat polyclonal anti-human Ngal antibody (R&D Systems), followed by incubation with TexasRed-labeled anti-goat IgG (Jackson ImmunoResearch, West Grove, PA, USA). The spillover of each signal was negligible.

Immunohistochemistry of Ngal and nephron markers

Mouse kidneys were fixed in 4% paraformaldehyde at 4°C for 12 hours and embedded in paraffin. Renal sections of 4 μ m were deparaffined, hydrated and incubated with 0.3% hydrogen peroxide. Antigen retrieval was performed by 0.05 mol L⁻¹ citrate buffer (pH 6.0) for 10min in a water bath heated at 100 °C (for Ngal, AQP1 and AQP2), or in a microwave oven (for THP). After cooling, the sections were incubated with 10% normal donkey or goat serum, followed by goat anti-mouse Ngal (1:300, R&D Systems), rabbit anti-AQP1 and AQP2 (1:200, Chemicon International, Temecula, CA, USA), or rabbit

anti-THP antibodies (1:200, Biomedical Technologies, Stoughton, MA, USA). Primary antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies and 3,3-diaminobenzidine tetrahydrochloride. Nuclei were counterstained with hematoxylin.

Statistical analysis

Results are expressed as mean \pm s.e. Student's t-test was carried out to compare two groups. Statistical significance was defined as $P < 0.05$.

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DISCLOSURE

The authors declare that they have no competing interests.

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SUPPLEMENTARY MATERIALS

Figure S1

Clinical course of cases with (a) lupus nephritis (Case 3, LN) and (b) crescentic glomerulonephritis (Case 4, CrescGN).

Figure S2

Low power fields showing expression of Ngal and nephron markers in unilateral ureteral obstruction (UUO) and contra-lateral kidneys.

Figure S3

High power fields of cortex showing expression of Ngal and nephron markers in UUO and contra-lateral kidneys.

Figure S4

High power fields of medulla showing expression of Ngal and nephron markers in UUO and contra-lateral kidneys.

Figure S5

Clinical course of a case with drug-induced interstitial nephritis (Case 5, IntN).

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Figure Legends

Figure 1

Urinary Ngal and albumin excretion in (a-c) A-ZIP/F-1 diabetic mice at 10 months of age and (d, e) diabetic mice at 8 weeks after streptozotocin (STZ) injection.

(a, d) Western blot of urine (25 μ l each) in individual mice and (b, c, e) urinary levels of Ngal and albumin (Alb) normalized by creatinine (Cr) are shown. (b) A-ZIP/F-1 mice including #1 excreted much larger volumes of urine than control FVB/N mice. In 3 control FVB/N mice, mean urinary Ngal/Cr ratio (\pm s.e.) was 42 ± 24 ng per mgCr. uNgal, urinary Ngal; rNgal, recombinant Ngal.

Figure 2

(a) Time course and (b) correlation of urinary Ngal and albumin excretion in streptozotocin (STZ)-induced diabetic mice.

(a) Urinary Ngal (uNgal) and albumin (uAlb) levels normalized by urinary creatinine (uCr) were examined before and at 4, 8, 12 weeks after STZ injection (mean \pm s.e.). #, $P < 0.05$ versus pre-STZ. Elevation of urinary Ngal levels was significant at 4, 8, 12 weeks if analyzed after log transformation. (b) Correlation between uNgal/uCr and uAlb/uCr. $r = 0.86$, $P < 0.001$, $n = 19$.

Figure 3

Ngal protein and mRNA expression in the serum, kidney and liver of STZ mice.

(a) Western blot of serum, whole kidney and liver at 8 weeks after induction of diabetes. Mice without STZ injection served as control (non-STZ). Equal amounts of serum (15 μ l), and protein (30 μ g) of whole kidney and liver were separated by electrophoresis. rNgal; recombinant Ngal. (b) Ngal mRNA expression levels were measured using real-time PCR and normalized by GAPDH expression ($n = 4$). The mean Ngal/GAPDH level in non-STZ whole kidney was arbitrary defined as 1.0. The whole kidneys at one day after unilateral ureteral obstruction (UUO) were also examined as a positive control. N.S., not significant.

Figure 4

Urinary excretion and tubular reabsorption of exogenously administered Ngal in STZ mice.

(a) Western blot of urinary Ngal detected either with anti-Ngal or with anti-His-tag antibody at 8 weeks after induction of diabetes. Urine samples were collected for 12 hours after His-tagged Ngal injection (i.p.) and 25 μ l aliquots of them were separated by electrophoresis. Of note, STZ mice excreted much more diluted urine

compared to non-STZ control mice. 1, endogenous Ngal (25 kDa, glycosylated); 2, His-tagged Ngal (21 kDa, unglycosylated). **(b)** Alexa Fluor 546-labeled Ngal was injected in STZ mice and kidneys were examined 30 min later. Arrows indicate Ngal protein distribution (in orange) which was homogeneous in non-STZ but was irregular and sparse in STZ mice. Top right panel shows selected area (in green) of positive fluorescence by a computer software. G, glomeruli. Magnification, x 20. **(c)** Quantitation of exogenous Ngal uptake in kidneys of STZ and non-STZ mice (n=4).

Figure 5

Reduction of urinary Ngal and albumin excretion by candesartan in STZ mice.

(a) Urinary Ngal (uNgal) and **(b)** albumin (uAlb) levels at 4 and 8 weeks after STZ injection and after one more week with candesartan (10 mg per kg per day, orally) or vehicle treatment (n=4).

Figure 6

Clinical course of cases with **(a)** minimal change disease (Case 1, MCD) and **(b)** lupus nephritis (Case 2, LN).

mPSL, methyl prednisolone; PSL, prednisolone; uProt, urinary protein excretion; s and uNgal, serum and urinary Ngal; BUN, blood urea nitrogen; sCr, serum creatinine; uNgal/uCr, urinary Ngal normalized by urinary creatinine; Ab, antibody.

Figure 7

Localization of Ngal protein expression in human and mouse kidneys.

Immunofluorescence of albumin **(a)**, green) and Ngal **(b)**, red) in a patient with minimal change disease (MCD, magnification 40 x). Arrows, co-localization of signals; asterisk, tubular lumen. Immunohistochemistry of Ngal **(c, d)**, aquaporin 1 **(e)**, AQP1) and Tamm-Horsfall protein **(f)**, THP) in the mouse kidney treated with unilateral ureteral obstruction (UUO; **d, f**) and in the contra-lateral kidney (UUN; **c, e**) at 1 day after operation (20 x). Serial sections were analyzed.

Figure 8

Ngal protein levels in UUO, UUN, serum and urine in mice with kidney obstruction.

(a) Western blot analysis of Ngal protein. N.D., not determined. **(b)** The mean Ngal concentrations in UUO, UUN, serum and urine (collected from dilated pelvis of the UUO side using a needle and syringe). Elevation of Ngal concentrations were statistically significant at day 1 in UUO or UUN kidneys and serum, and at

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Source and changes of urinary Ngal in renal injury

day 3 in urine ($P < 0.01$ by unpaired t test, $n = 3$) compared to day 0 (no operation).

Table 1

Blood glucose, urea nitrogen, creatinine levels, body weight and blood pressure in STZ diabetic mice before and after candesartan treatment

	Vehicle		Candesartan	
	Before	After	Before	After
Blood Glucose (mg per 100 ml)	598±2	593±6	600±3	591±5
HbA1c (%)	11.7±0.5	N.D.	12.5±0.2	N.D.
Blood Urea Nitrogen (mg per 100 ml)	N.D.	50±2	N.D.	54±1
Serum Creatinine (mg per 100ml)	N.D.	0.13±0.01	N.D.	0.11±0.01
Body Weight (g)	23.8±0.9	23.5±0.7	22.4±0.5	22.8±0.5
Systolic Blood Pressure (mmHg)	104±2	105±0.9	103±1	100±1
Diastolic Blood Pressure (mmHg)	55±2	56±1	50±1	49±2

Treatment with candesartan did not significantly alter these parameters. Blood urea nitrogen and serum creatinine levels in non-STZ control mice were 23±3 and 0.09±0.02 mg per 100 ml, respectively. Blood was drawn when mice were fed ad libitum. N.D., not determined.

Table 2

Changes of urinary biomarker levels in case 5, who had interstitial nephritis

Days after Admission	9	37	Fold Difference ^a	Normal Value
uNgal/uCr (μg per gCr)	256.6	25.4	10.1	^b <10 μg L ⁻¹
uProt/uCr (g per gCr)	0.381	0.052	7.3	<0.15 g day ⁻¹
uβ2MG/uCr (mg per gCr)	18.2	2.8	6.5	<0.3 mg L ⁻¹
uα1MG/uCr (mg per gCr)	46.4	17.2	2.7	<15 mg L ⁻¹
sNgal (μg L ⁻¹)	224	90	2.5	^b <106 μg L ⁻¹
uNAG/uCr (U per gCr)	21.1	8.7	2.4	<7 U L ⁻¹
BUN (mg per 100 ml)	15	11	1.4	<22 mg per 100 ml
sCr (mg per 100 ml)	1.2	1.0	1.2	^c <1.1 mg per 100 ml

u and sNgal, urinary and serum Ngal; uCr, urinary creatinine; Prot, protein; MG, microglobulin; NAG, N-acetyl-beta-D-glucosaminidase.

^aFold difference between days 9 and 37.

^bValue suggested by ELISA kit supplier.

^c<1.1 for male and <0.8 for female.

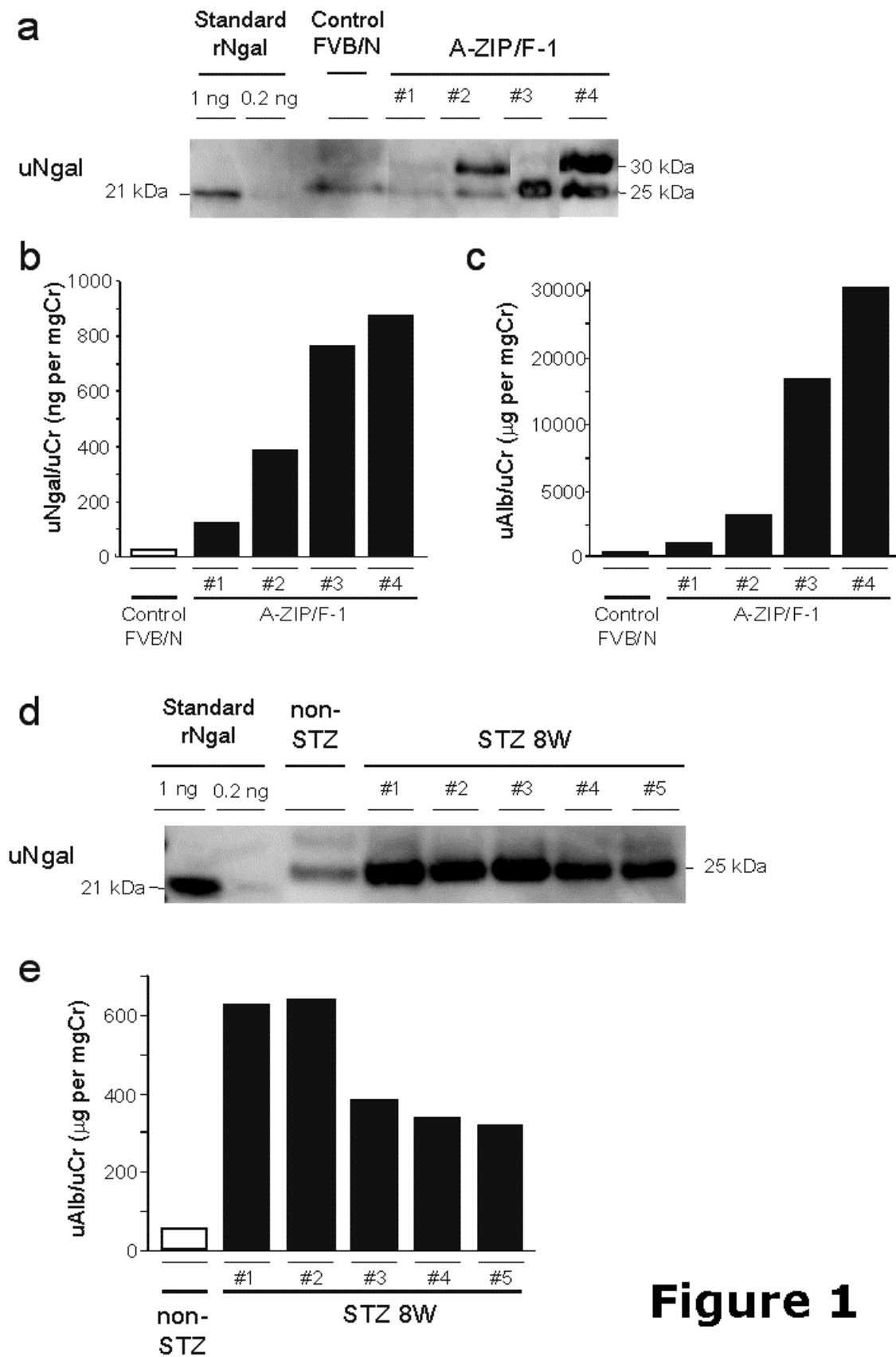
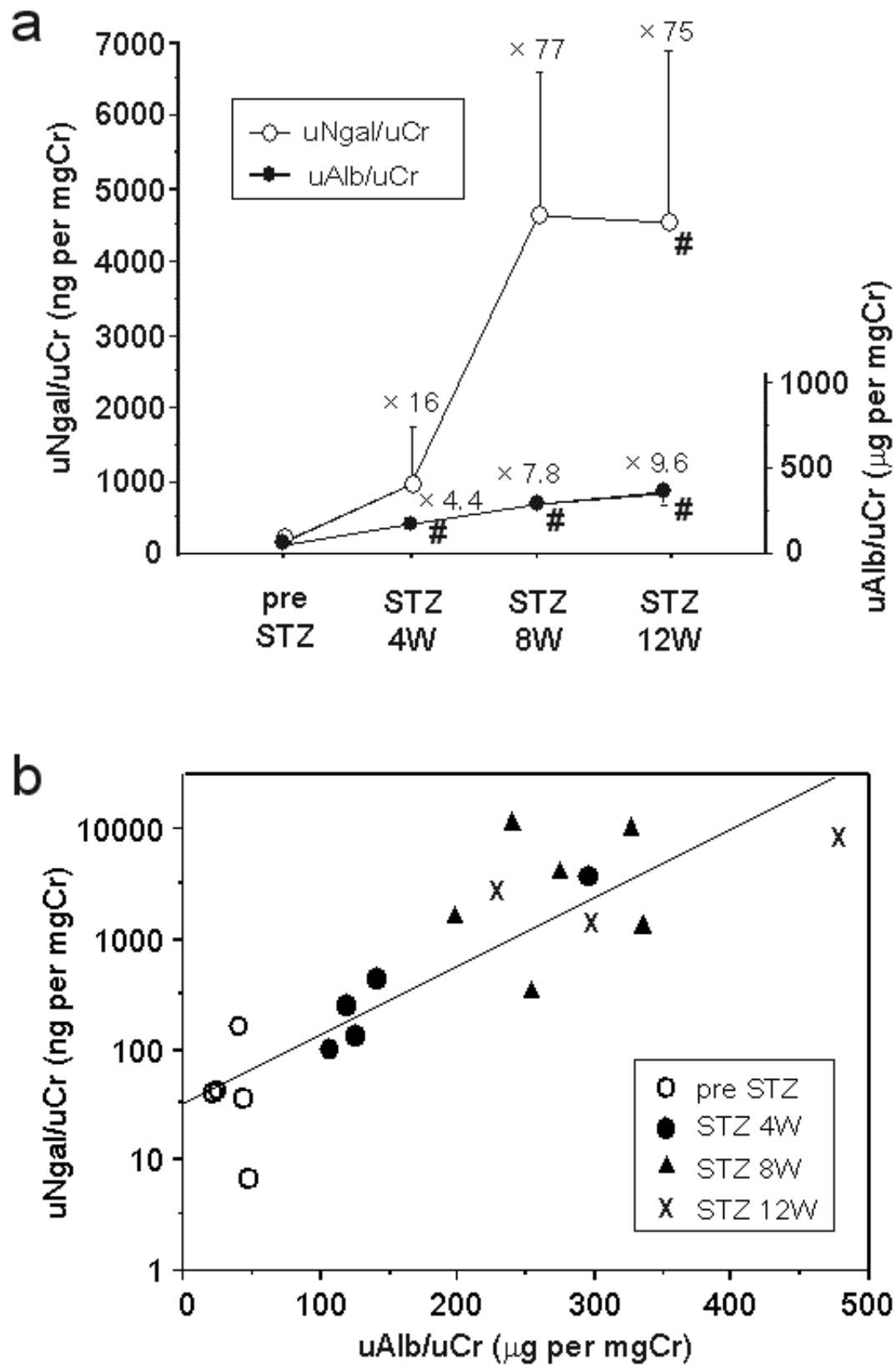
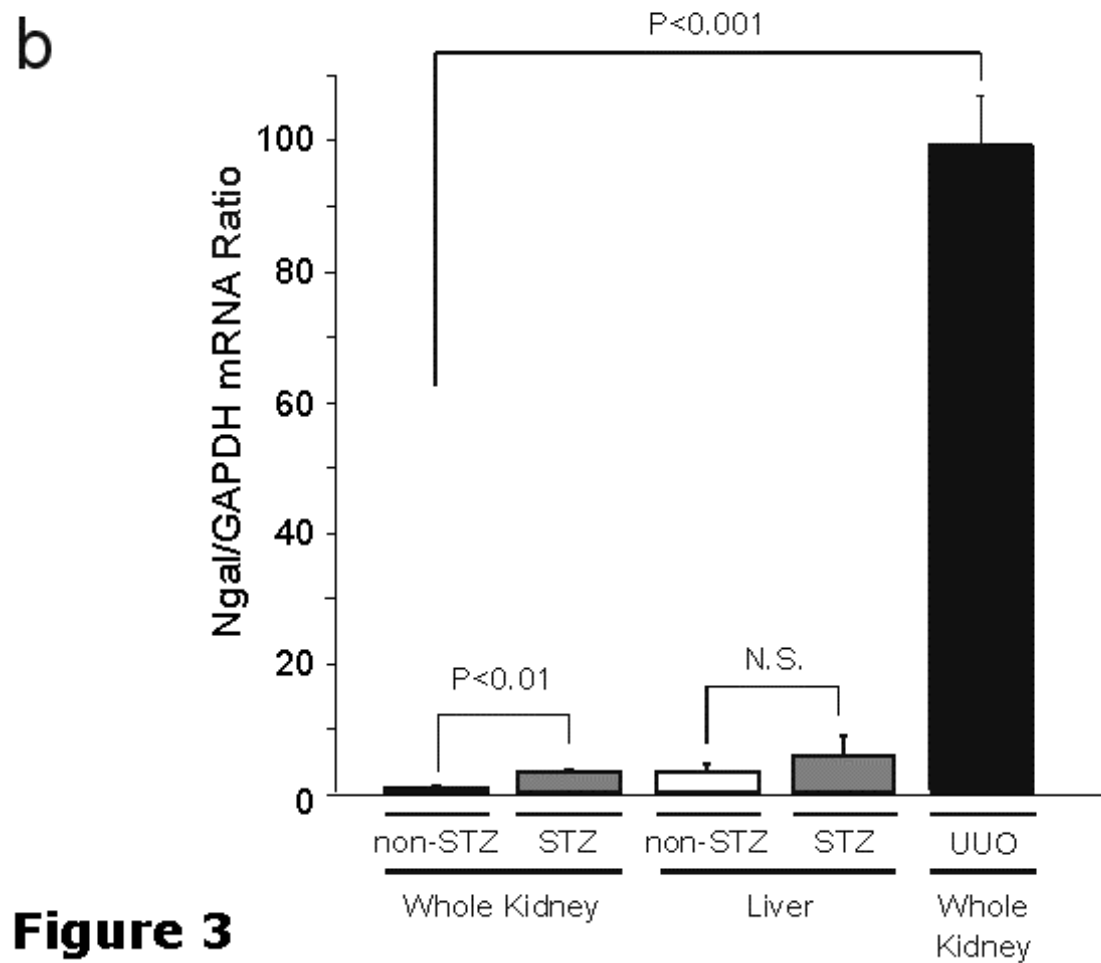
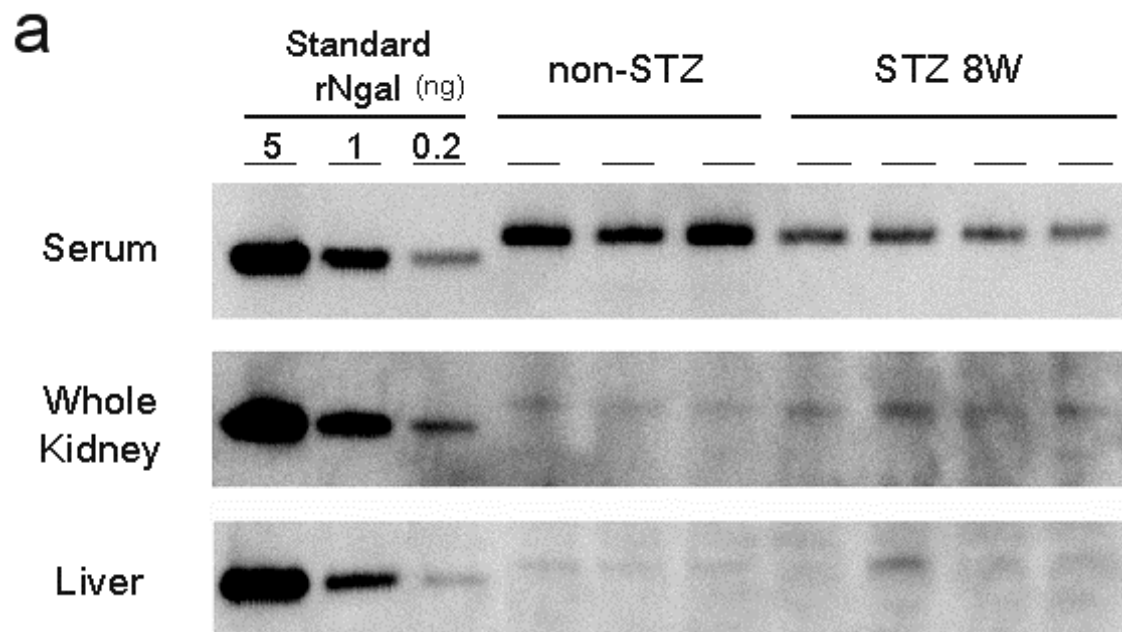


Figure 1

Figure 2





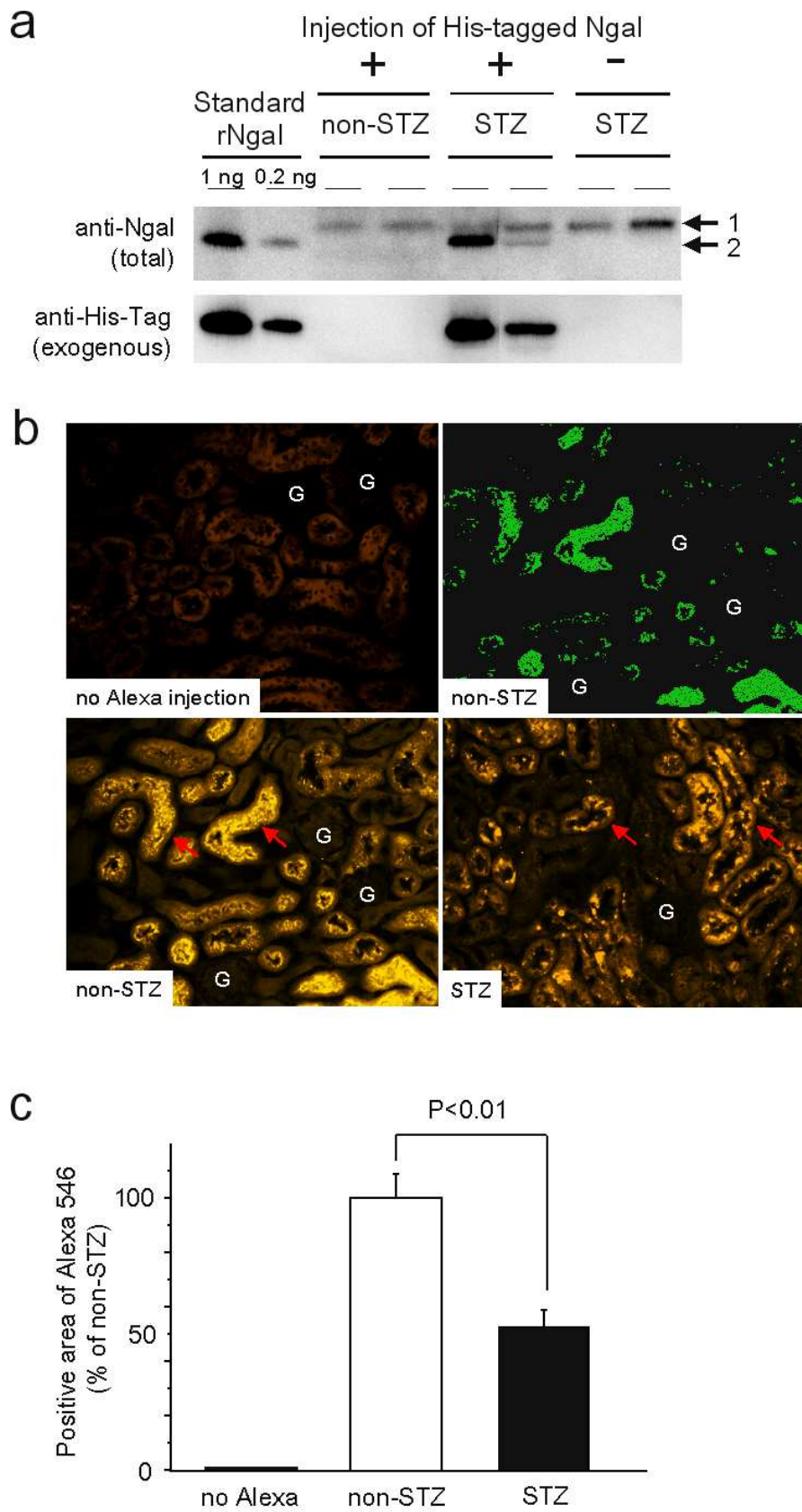
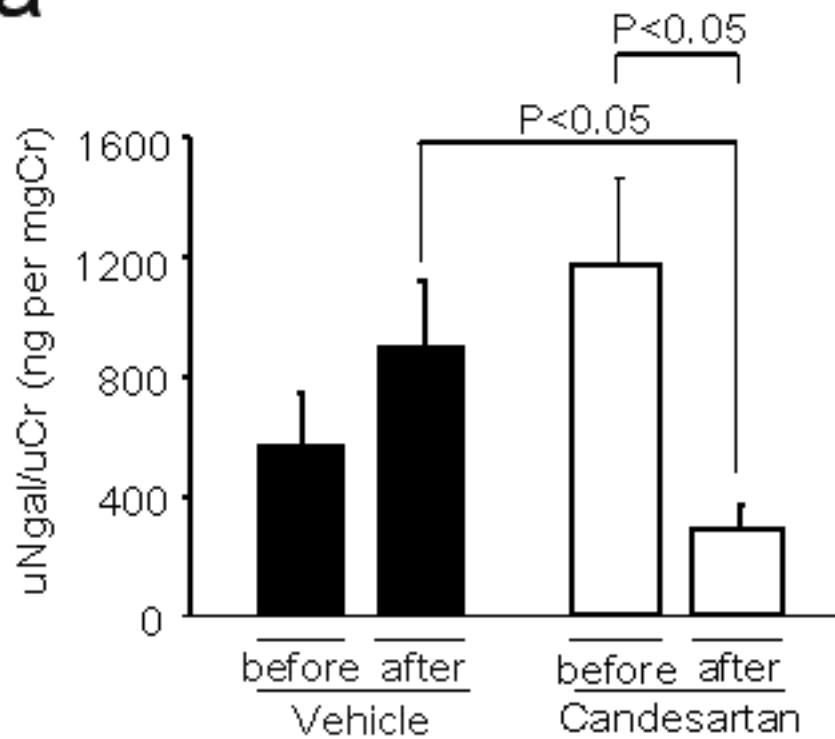


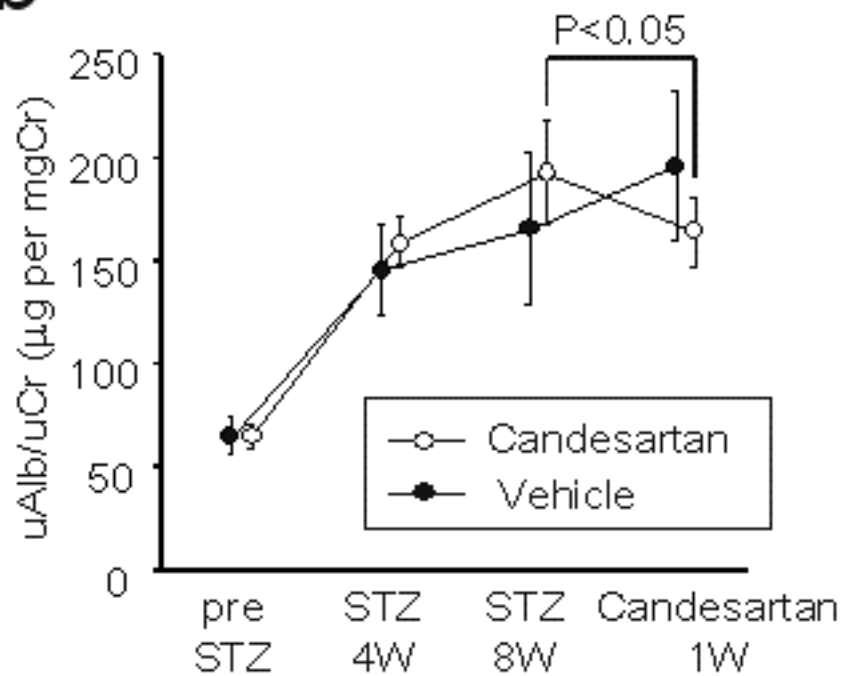
Figure 4

Figure 5

a



b



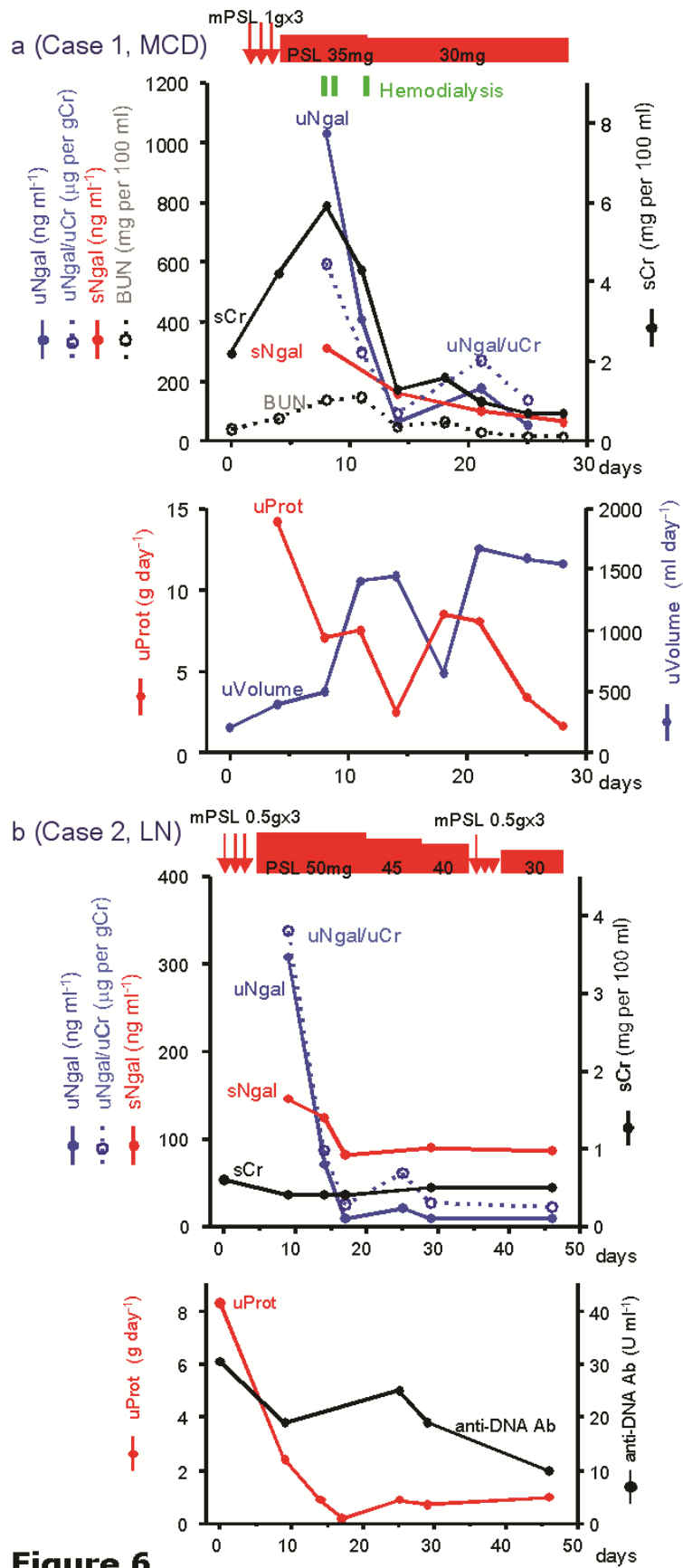


Figure 7

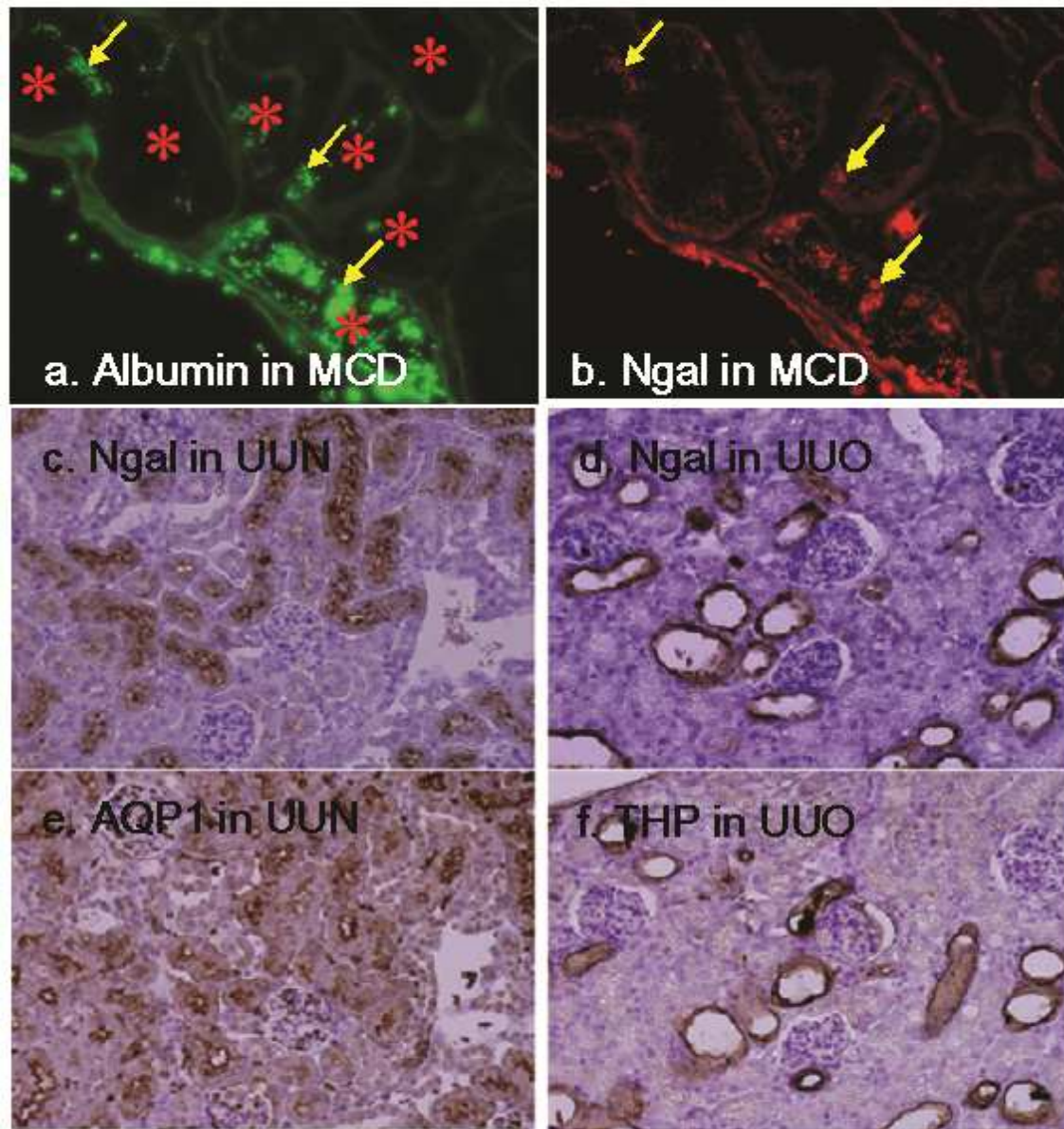
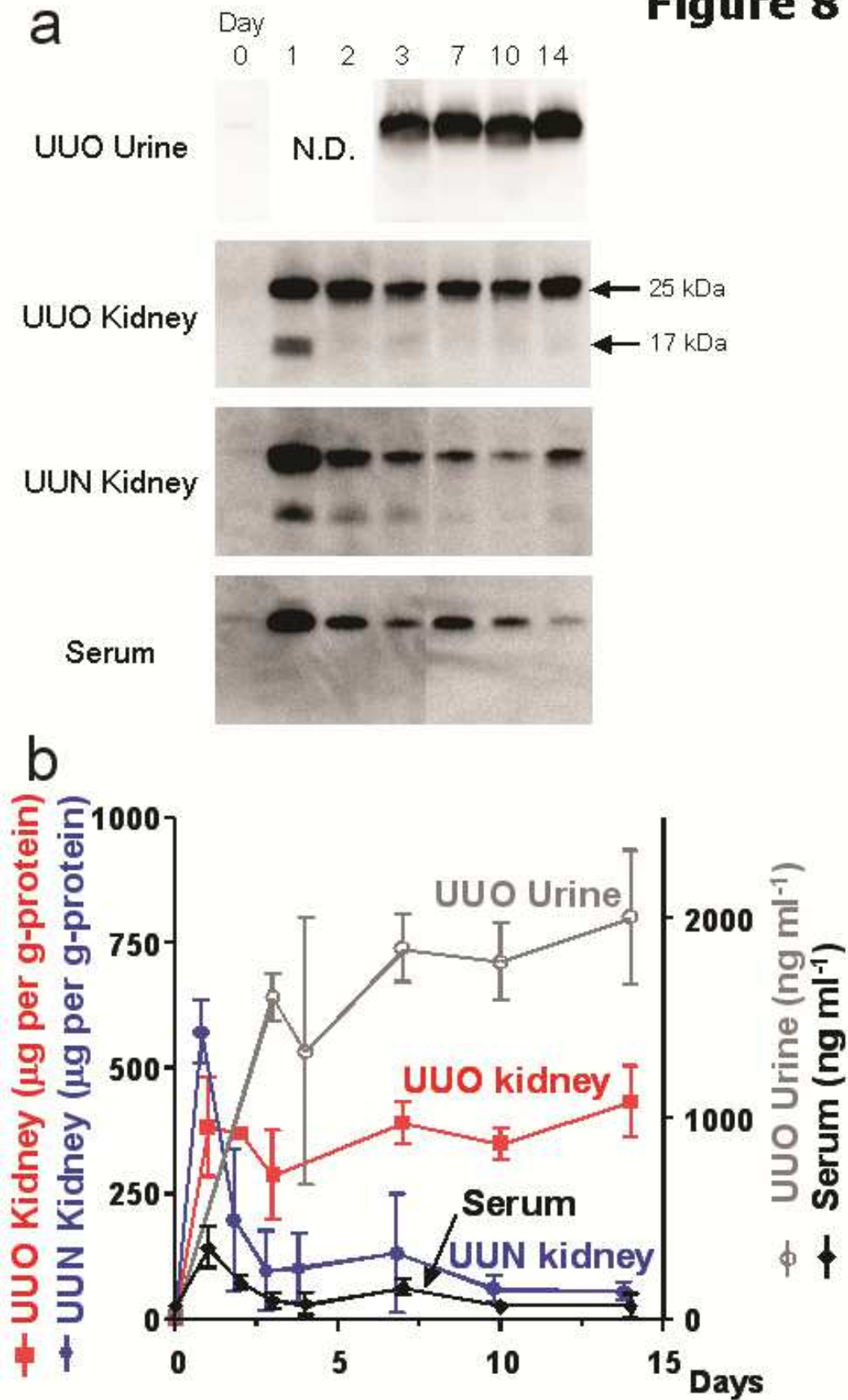


Figure 8

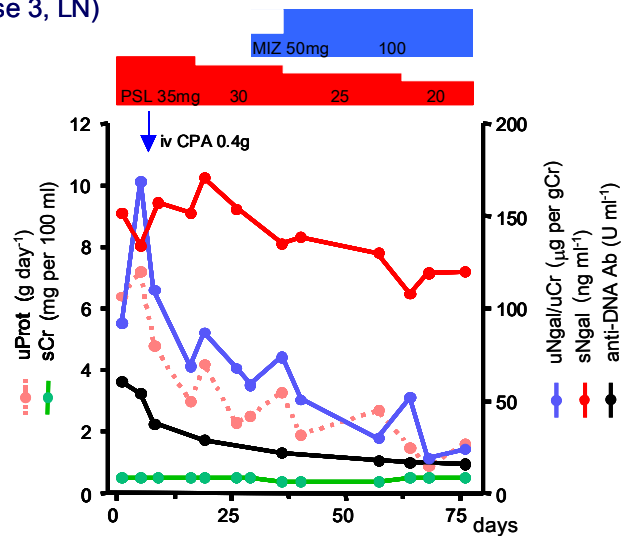


Supplementary Figure S1

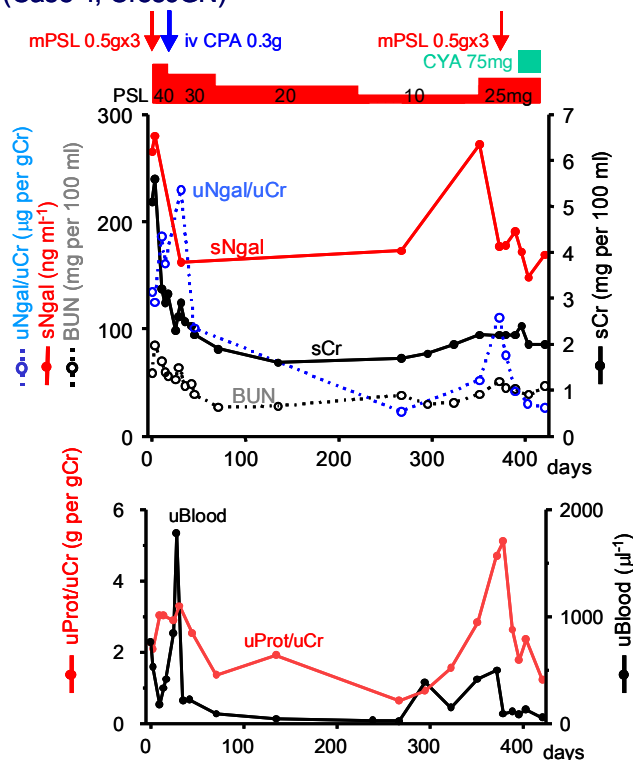
Clinical course of cases with (a) lupus nephritis (Case 3, LN) and (b) crescentic glomerulonephritis (Case 4, CrescGN).

mPSL, methyl prednisolone; PSL, prednisolone; uProt, urinary protein excretion; s and uNgal, serum and urinary Ngal; BUN, blood urea nitrogen; sCr, serum creatinine; uNgal/uCr, urinary Ngal normalized by urinary creatinine; Ab, antibody. CPA, cyclophosphamide; MIZ, mizoribine; CYA, cyclosporine A; uBlood, extent of hematuria determined by flowcytometer.

a (Case 3, LN)



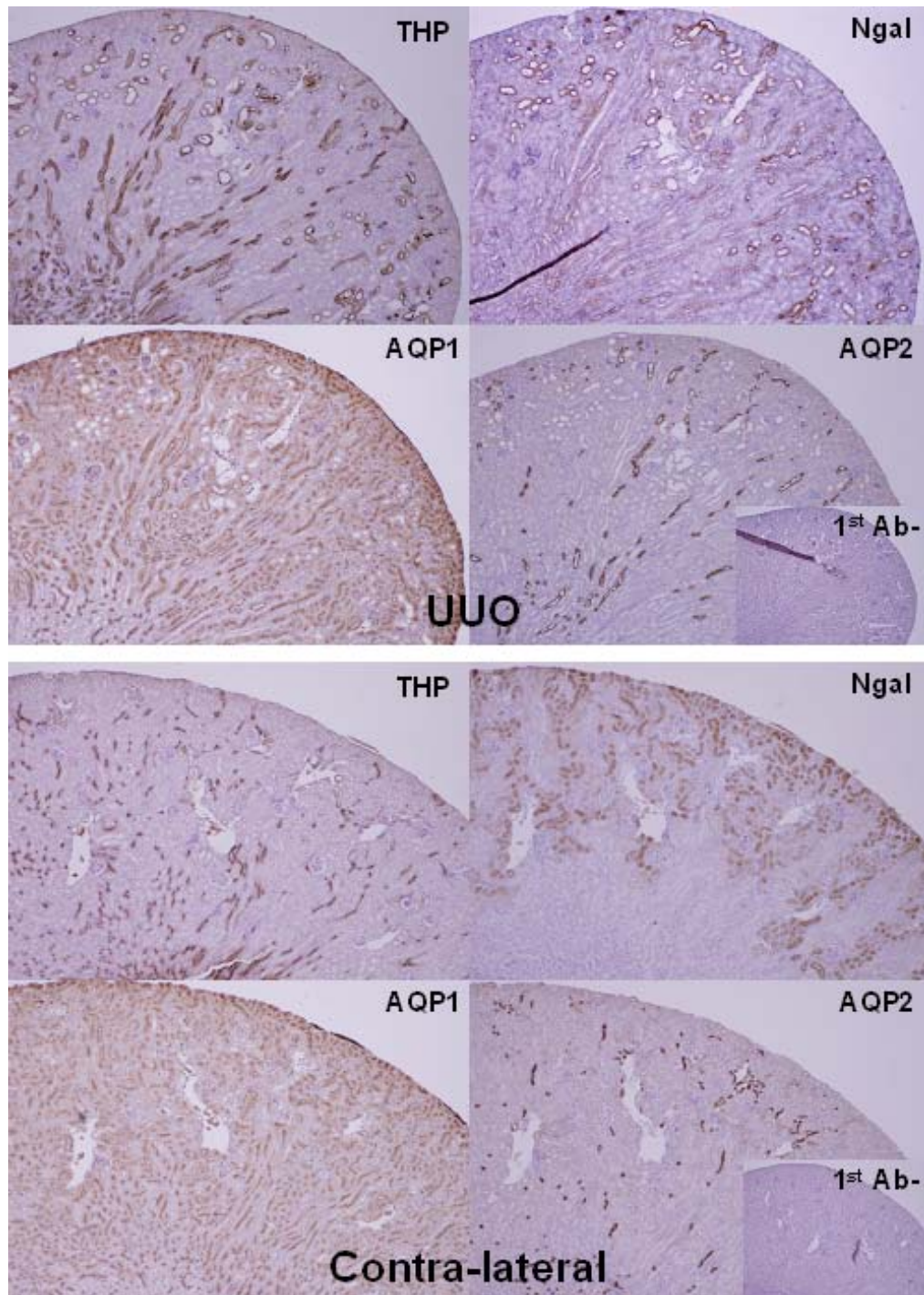
b (Case 4, CrescGN)



Supplementary Figure S2

Low power fields (4 x) showing expression of Ngai and nephron markers in unilateral ureteral obstruction (UUO, top) and contra-lateral kidneys (bottom) (in brown) after 1 day of ureter ligation.

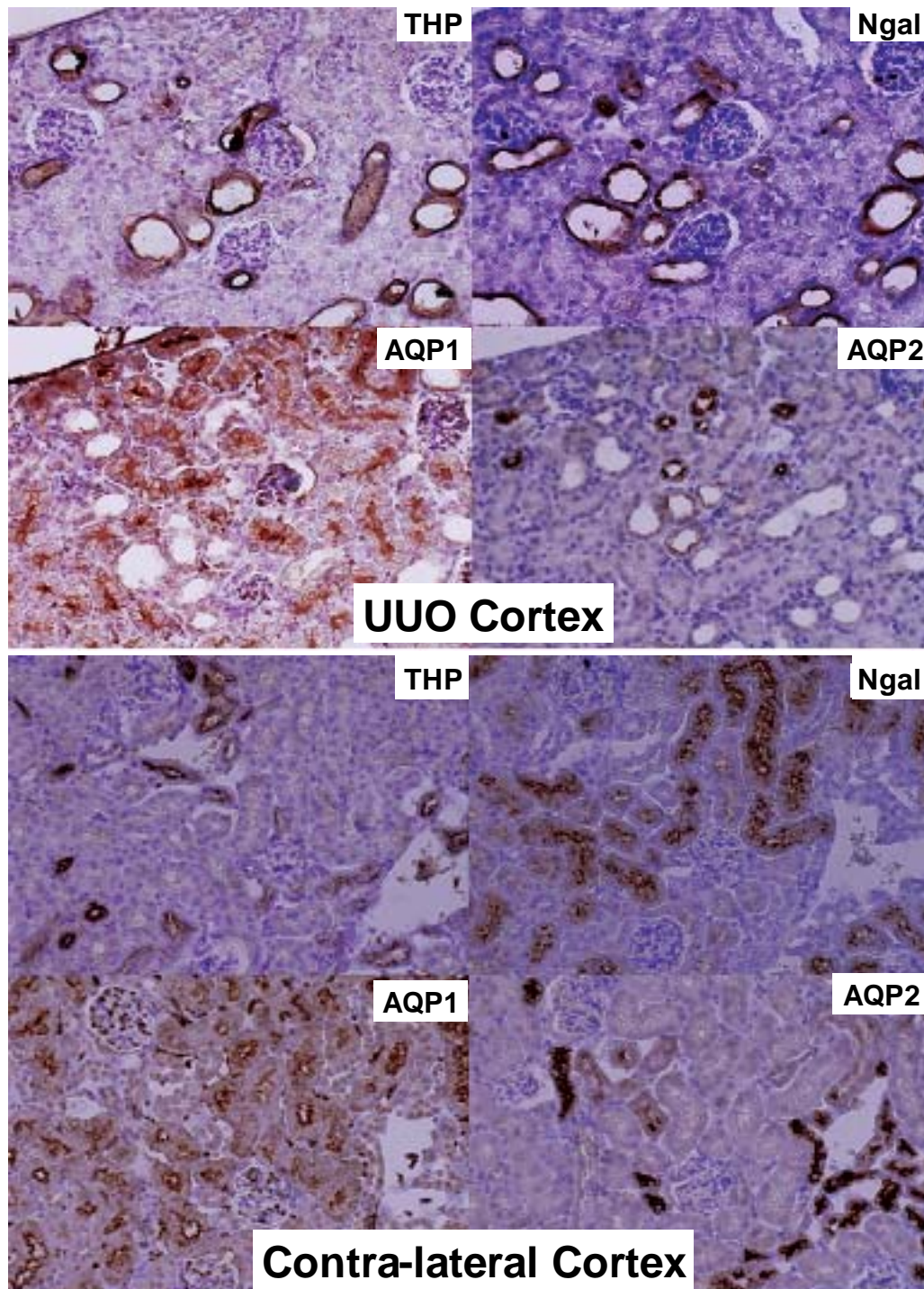
AQP, Aquaporin; THP, Tamm-Horsfall protein. 1st Ab-, without 1st antibody (negative control for immunohistochemistry, inset).



Supplementary Figure S3

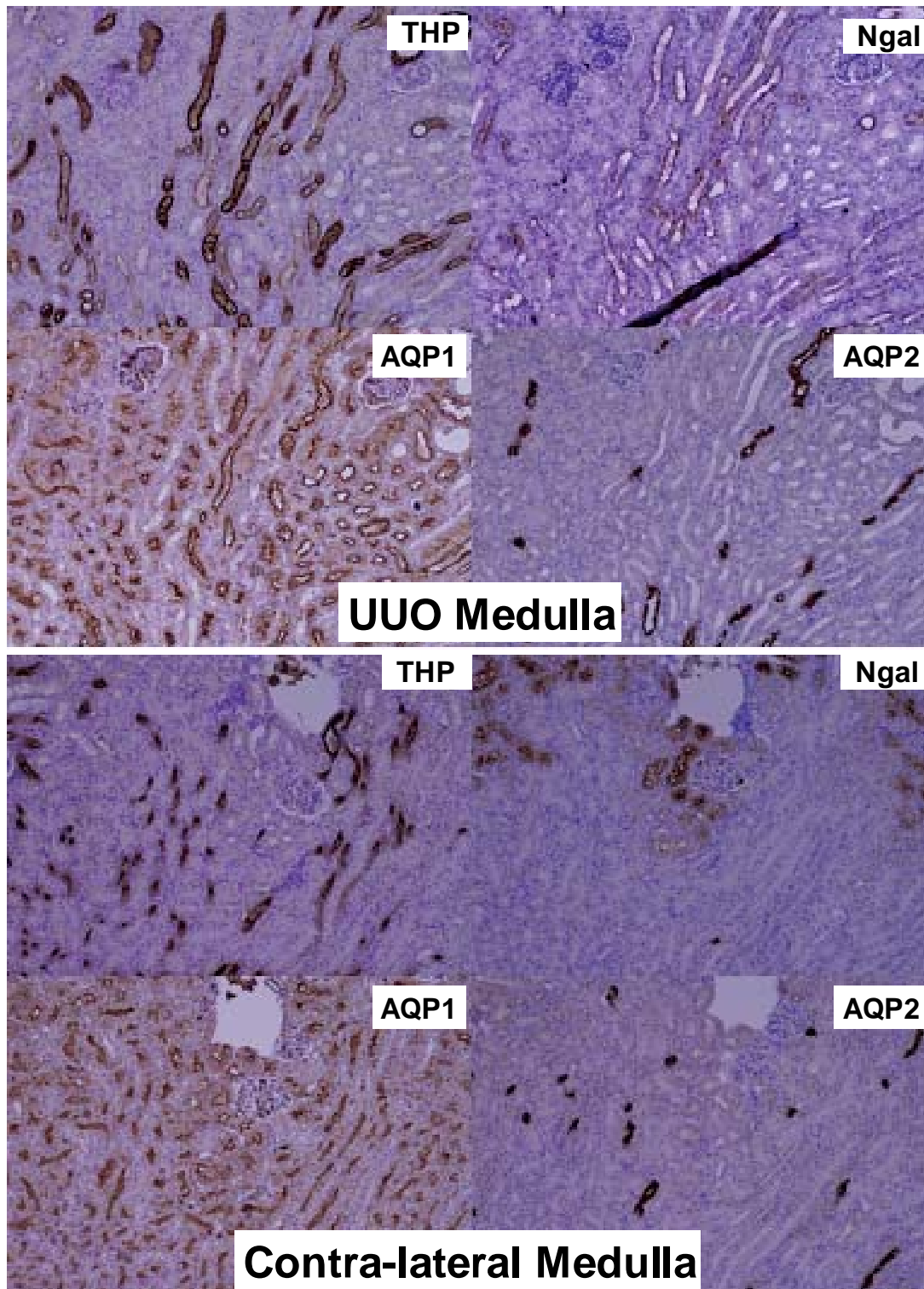
High power fields of cortex (20 x) showing expression of Ngal and nephron markers in UUO and contra-lateral kidneys.

Distribution of Ngal co-localized well with THP in UUO kidneys, whereas with AQP1 in contra-lateral kidneys.



Supplementary Figure S4

High power fields of medulla (10 x) showing expression of Ngal and nephron markers in UUO and contra-lateral kidneys.



Supplementary Figure S5

Clinical course of a case with drug-induced interstitial nephritis (Case 5, IntN).

Changes of urinary biomarkers are shown in percentage by assigning 100% and 0% for the levels on days 9 and 37, respectively. mPSL, methyl prednisolone; PSL, prednisolone; uProt, urinary protein excretion; s and uNgal, serum and urinary Ngal; BUN, blood urea nitrogen; sCr, serum creatinine; uNgal/uCr, urinary Ngal normalized by urinary creatinine; MG, microglobulin; NAG, N-acetyl-beta-D-glucosaminidase

(Case 5, IntN)

